Current Protocols in Cytometry

May 5, 2008

Dr. Jaco Kraan
Department of Medical Oncology
Daniel den Hoed Cancer Center
Erasmus MC—Univ. Medical Center Rotterdam
P.O. Box 5201
3008 AE Rotterdam
THE NETHERLANDS

Tel: (31-10) 439-1418
Fax: (31-10) 439-1005
j.kraan@erasmusmc.nl

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1. Tables, (a) In each table, please add a footnote to clarify exactly how to determine the exact volume of each antibody to add, as the tables are somewhat hard to interpret (e.g., the units of measurement corresponding to the numbers are not specified). Also, please clarify exactly what the numbers "plus kappa and lambda" mean in these tables.

   (b) In each table the word "query" was accompanied by an asterisk, which we have deleted because it was not accompanied by an explanation. If you wish, please give an explanation of these asterisks and we will insert it in the form of a lettered footnote.

2. Basic Protocol materials list: (a) We cross-referenced Support Protocol 2 for the CSF sample; is this correct?

   (b) Correct that the 1% paraformaldehyde is calculated on a weight/volume (w/v) basis, as PFA is a solid?

   (c) Please suggest a supplier for the "flow buffer" and "rinse buffer."

3. Basic Protocol, (a) step 1: Correct that the PBS is added to the whole CSF sample obtained in Support Protocol 2 (i.e., 2 ml of the fluid itself, plus the stabilization medium)?

   (b) steps 1 and 7: At what temperatures are these centrifugations performed?

   (c) step 10: By "100.000 cells" do you mean "one hundred thousand" (i.e., should the decimal point be replaced with a comma in an English-language publication)?

4. Support Protocol 1, generally: We have added some bold italic headers to set off the different procedures; please feel free to edit or relocate these as you deem fit.

5. Support Protocol 1, "Additional Materials": Is it correct to cite Strategic Planning and Tables 6.25.1-6.25.5 for <u>both</u> the surface and the cytoplasmic antibody cocktails?

6. Support Protocol 1, (a) step 1: Correct that this protocol starts with 100 µl of the CSF sample as prepared in Support Protocol 2?

   (b) steps 5 9, and 14: At what temperature is each centrifugation performed?

7. Alternate Protocol, (a) steps 1 and 7: At what temperature are these centrifugations performed?

8. Acknowledgements: Per Current Protocols style, we have created an Acknowledgement section to accommodate information that was originally presented along with the author list. Is this correctly worded? If not, please edit as you see fit.

9. Literature Cited: Bierman and Giglio (2005), Keldsen et al. (1996), and Van Acker et al. (2001) are not cited in the text; please find appropriate locations at which to cite.

Flow Cytometric Immunophenotyping of Cerebrospinal Fluid

Jaco Kraan,[1] Jan W. Gratama,[1] Corinne Haioun,[2] Alberto Orfao,[3] Anne Plonquet,[4] Anna Porwit,[5] Sandra Quijano,[3] Maryalice Stetler-Stevenson,[6] Dolores Subira,[7] and Wyndham Wilson[8]

[1]Department of Medical Oncology, Erasmus Medical Center, Rotterdam, The Netherlands
[2]Service d'Hématologie Clinique, Hospital Henri Mondor, Créteil, France
[3]CSIC, University of Salamanca, Salamanca, Spain
[4]Immunologie Biologique, Hospital Henri Mondor, Créteil, France
[5]Department of Pathology, Karolinska University Hospital, Stockholm, Sweden
[6]Flow Cytometry Unit, National Institutes of Health, Bethesda, Maryland
[7]Department of Hematology, Jiménez Díaz Foundation, Madrid, Spain
[8]Center for Cancer Research, National Institutes of Health, Bethesda, Maryland

ABSTRACT

Leptomeningeal disease is an important adverse complication occurring in patients with B and T cell lymphomas and acute leukemias of lymphoid and myeloid origin. Recent reports suggest that multiparameter flow cytometry immunophenotypic assessment of spinal fluid samples could improve the efficiency of detection of CNS involvement, due to its high specificity and greater sensitivity. However, spinal fluid samples are frequently paucicellular with a rapidly decreasing cell viability. Staining of spinal fluid therefore requires dedicated sample storage/transport, staining and preparation protocols. The Basic Protocol in this unit outlines a consensus multiparameter (3- to 8-color) flow cytometry immunophenotypic protocol for the evaluation of CNS involvement of cerebrospinal fluid (CSF) samples by neoplastic cells. A Support Protocol describing the simultaneous assessment of surface and cytoplasmic antigens is also provided. Finally, in the Alternate Protocol, we describe a method to calculate absolute numbers of both normal and pathological cell subpopulations by adding counting beads to the assay. *Curr. Protoc. Cytom.* 45:6.25.1-6.25.16. © 2008 by John Wiley & Sons, Inc.

Keywords: CSF • cerebrospinal fluid • diagnosis • flow cytometry • immunophenotyping • multi-color • leukemia

INTRODUCTION

Leptomeningeal disease is considered an important adverse complication occurring in patients with B and T cell lymphomas, as well as in patients with acute leukemias of lymphoid and myeloid origin. Cytological assessment of cerebrospinal fluid samples for neoplastic cells is essential for the diagnosis of central nervous system (CNS) involvement, particularly among patients suffering from myeloid and B cell neoplasias. Although a positive conventional cytological analysis of a spinal fluid sample is highly specific for CNS involvement, a relatively high rate of false negative results (up to 60% of the cases) has been associated with this technique. Recent reports suggest that multiparameter flow cytometry immunophenotypic assessment of spinal fluid samples could improve the efficiency of detection of CNS involvement, due to the high specificity and greater sensitivity of this technique. However, spinal fluid samples are frequently paucicellular and contain a limited number of cells with a rapidly decreasing viability. Therefore, conventional immunophenotypic protocols applied to peripheral blood, bone

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marrow, and lymphoid tissue samples are suboptimal. Staining of spinal fluid requires dedicated sample storage/transport, staining, and preparation protocols.

The Basic Protocol in this unit outlines a consensus multiparameter flow cytometry immunophenotypic protocol for the evaluation of cerebrospinal fluid (CSF) for neoplastic cells to detect CNS involvement. The protocol is designed for 3 to 8 colors, depending on the number of parameters available on the flow cytometer used. The method can be applied in freshly obtained or stabilized samples. Samples should be processed within 1 hr after lumbar puncture; otherwise they will need to be stabilized to avoid deterioration of cells due to the rapid in vitro cytotoxic effects of CSF on leukocytes. A Support Protocol describing the simultaneous assessment of surface and cytoplasmic antigens is also provided. Finally, in the Alternate Protocol, we describe a method to calculate absolute numbers of both normal and pathological cell subpopulations by adding counting beads to the assay.

STRATEGIC PLANNING

Selection of Antibody Combinations

Assuming appropriate and standardized instrument setup (see Chapter 1), selection of reagents and their pairing with appropriate fluorochromes are key factors in assuring appropriate detection sensitivity. Each staining combination should be designed to address a clearly stated objective and answer one or more queries. CSF samples are associated with extremely low cell numbers; therefore, a two-step approach is suggested. In the first step, one-third of the sample will be analyzed using a "screening" tube, which in most cases will answer the clinical question. When the first step is not conclusive, the process should be repeated with the remaining CSF volume and the same reagent combination, to increase the sensitivity of the assay. In contrast, if a pathological population is identified, a complete phenotypic characterization will follow in the second step, using antibody reagent panels adapted to the number of cells and previous histological and/or immunophenotypic diagnosis or suspected diagnosis.

Antibodies to cytoplasmic antigens may be of help in identifying and characterizing neoplastic cells in CSF. However, increased cell loss is a significant drawback associated with intracellular staining; therefore, its use should be limited to those cases where it is essential to reach the immunological conclusion.

Approaches based on the simultaneous use of a high number of multiple monoclonal antibodies per tube, if appropriately chosen, significantly add to the diagnostic power of the assay and are therefore recommended.

Examples of Staining Panels for Screening Tube(s)

Panels suitable for the identification of aberrant, leukemia, or lymphoma cells in CSF are designed depending on the clinical question and available instrumentation, and examples are presented in Tables 6.25.1-6.25.5 (step 1, screening; see above). When neoplastic cells are present, a complete phenotypic characterization with additional reagent combinations may be performed. Panels of reagents must be empirically tested to assure that the signal intensities are as expected. Dimly expressed antigens must be appropriately detected, and the occurrence of undesired interactions between reagents must have been excluded in advance. Fluorescence intensity of each reagent should be tested on normal blood or bone marrow samples containing positive and negative cell populations of interest. Optimally, each reagent should be individually titrated by testing a range of different concentrations. The resulting data are then inspected to select the titer resulting in the optimal signal-to-noise ratio, without increase in intensity for the negative population (see *UNIT 4.1*).

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Table 6.25.1 Eight-Color Immunophenotyping Panel[a]

Laser		Blue (488 nm)				Red (635 nm)		Violet (407 nm)	
Query	Tube no.	FITC	PE	PCX	PEC7	APC	APC7	PB	PO
Unknown	1	8 + λ	56 + κ	4+19	3	38	14	20	45
B	1	λ	CDX/IgX	19	10	κ	45	20	45
T	1	8	56	4	3	7	5	2	45
AL	1	34	7	19	33	10	DR	20	45
MM	1	138	56	19	38	20	3	45	CDX

[a]Abbreviations: AL, acute leukemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; APC, allophycocyanin; APC7, APC-Cy7; B, B cell lymphoma; CLL, chronic lymphocytic leukemia; FITC, fluorescein isothiocyanate; MM, multiple myeloma; PB, Pacific blue; PE, phycoerythrin; PE-TR, PE-Texas red; PCX, PE-cyanin (Cy)5 (PEC5) *or* PE-Cy5.5 *or* peridinin chlorophyll protein (PerCP) *or* PerCP.CY5.5; PEC7, PE-Cy7; PO, Pacific orange; T, T cell lymphoma.

Table 6.25.2 Six-Color Immunophenotyping Panel[a]

Laser		Blue (488 nm)				Red (635 nm)	
Query	Tube no.	FITC	PE	PCX	PEC7	APC	APC7
Unknown	1	8 + λ	56 + κ	4+19	3	38	45
B	1	λ	CDX/IgX	19	10	κ	45
T	1	5	7	45	4	8	3
AL	1	34	7	45	33	10	19
ALL	1	5	7	45	10	34	19
AML	1	34	117	45	33	133	DR
MM	1	38	138	45	56	20	19

[a]Abbreviations: AL, acute leukemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; APC, allophycocyanin; APC7, APC-Cy7; B, B cell lymphoma; CLL, chronic lymphocytic leukemia; FITC, fluorescein isothiocyanate; MM, multiple myeloma; PB, Pacific blue; PE, phycoerythrin; PE-TR, PE-Texas red; PCX, PE-cyanin (Cy)5 (PEC5) *or* PE-Cy5.5 *or* peridinin chlorophyll protein (PerCP) *or* PerCP.CY5.5; PEC7, PE-Cy7; PO, Pacific orange; T, T cell lymphoma.

To evaluate the presence of reagent interactions, a sample prepared with all reagents can be compared with a sample prepared with a series of preparations each lacking one of the component reagents (fluorescence-minus-one, or FMO controls; *UNIT 1.14*), as well as with a second series containing each single reagent independently. Significant interactions between reagents will be seen by alterations of signal intensity or patterns visualized by two-parameter dot plots for each series. Once reagent combinations and titers are selected, a cocktail of reagents can be created to allow more efficient delivery of the reagents and to minimize the chance of errors in reagent addition.

Instrument Setup and Color Compensation

Appropriate instrument setup (*UNIT 1.3*) and color compensation (*UNIT 1.14*) are essential to the success of multicolor flow cytometry. Software compensation, either on the instrument or off-line, is the only viable option for dealing with compensation in multicolor (>3-color) flow cytometry. A series of samples stained with each individual fluorochrome of interest should be used to set the compensation, and positive population should be

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Table 6.25.3 Five-Color Immunophenotyping Panel[a]

Laser		Blue (488 nm)				Red (635 nm)
Query	Tube no.	FITC	PE	PE-TR	PEC5	APC7
Unknown	1	8 + λ	56 + κ	45	4+19	3
	2	5	10	45	19	22
B	1	λ	κ	19	45	CDx
	2	20	10	45	19	CDx
T	1	8	7	5	4	3
	2	2	56	5	7	3
AL	1	34	10	45	7	19
ALL	1	5	10	45	7	19
AML	1	34	117	45	33	DR
MM	1	38	138	45	56	19

[a]Abbreviations: AL, acute leukemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; APC, allophycocyanin; APC7, APC-Cy7; B, B cell lymphoma; CLL, chronic lymphocytic leukemia; FITC, fluorescein isothiocyanate; MM, multiple myeloma; PB, Pacific blue; PE, phycoerythrin; PE-TR, PE-Texas red; PCX, PE-cyanin (Cy)5 (PEC5) *or* PE-Cy5.5 *or* peridinin chlorophyll protein (PerCP) *or* PerCP.CY5.5; PEC7, PE-Cy7; PO, Pacific orange; T, T cell lymphoma.

Table 6.25.4 Four-Color Immunophenotyping Panel[a]

Laser		Blue (488 nm)			Red (635 nm)
Query	Tube	FITC	PE	PCX	APC
Unknown	1	8 + λ	56 + κ	4 +19	3
	2	5	10	45	19
B	1	λ	CDX/IgX	19/20	κ
	2	20	10	45	19
T	1	4	8	45	3
	2	5	56	3	7
AL	1	19	34	45	10
ALL	1	7	10	45	34
AML	1	14	33	45	34
MM	1	56	138	45	38

[a]Abbreviations: AL, acute leukemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; APC, allophycocyanin; APC7, APC-Cy7; B, B cell lymphoma; CLL, chronic lymphocytic leukemia; FITC, fluorescein isothiocyanate; MM, multiple myeloma; PB, Pacific blue; PE, phycoerythrin; PE-TR, PE-Texas red; PCX, PE-cyanin (Cy)5 (PEC5) *or* PE-Cy5.5 *or* peridinin chlorophyll protein (PerCP) *or* PerCP.CY5.5; PEC7, PE-Cy7; PO, Pacific orange; T, T cell lymphoma.

as bright as the brightest signal planned, to evaluate for each fluorochrome. For tandem fluorochromes, it is important to separately compensate for each individual reagent containing a tandem fluorochrome, as the spectral emission properties can vary from lot to lot, among manufacturers, and over time.

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Table 6.25.5 Three-Color Immunophenotyping Panel[a]

Laser		Blue (488 nm)		
Query	Tube	FITC	PE	PCX
Unkown/B lymphoma	1	3+14	19	45
	2	λ	κ	19/20
	3	19	10	45
CLL	3	19	5	45
MM	3	138	56	45
T lymphoma	1	3+14	5	45
	2	8	4	3
	3	DR	5	7
AL	1	34	10	45

[a]Abbreviations: AL, acute leukemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; APC, allophycocyanin; APC7, APC-Cy7; B, B cell lymphoma; CLL, chronic lymphocytic leukemia; FITC, fluorescein isothiocyanate; MM, multiple myeloma; PB, Pacific blue; PE, phycoerythrin; PE-TR, PE-Texas red; PCX, PE-cyanin (Cy)5 (PEC5) *or* PE-Cy5.5 *or* peridinin chlorophyll protein (PerCP) *or* PerCP.CY5.5; PEC7, PE-Cy7; PO, Pacific orange; T, T cell lymphoma.

IMMUNOPHENOTYPIC ANALYSIS OF WHITE BLOOD CELLS WITH MULTIPLE MONOCLONAL ANTIBODIES TO SURFACE ANTIGENS

BASIC PROTOCOL

The following method was developed to minimize sample manipulation and maximize recovery of white blood cells in CSF. The method describes a standardized multiparameter flow cytometry approach to identify normal and neoplastic cells by using logical and sequential gating strategies.

Materials

Phosphate-buffered saline (PBS), pH 7.6 (*APPENDIX 2A*)
Cerebrospinal fluid (CSF) sample (Support Protocol 2)
Antibody cocktail (see Strategic Planning and Tables 6.25.1-6.25.5)
Phosphate-buffered saline (PBS), pH 7.6) (*APPENDIX 2A*) containing 1% (w/v) paraformaldehyde (PFA)
Rinse buffer (?Supplier?)
Flow buffer (?Supplier?)

Centrifuge
12 × 75–mm polystyrene tubes
Flow cytometer

Label cell surface antigens

1. Add 3 to 5 ml PBS to the entire CSF sample obtained in Support Protocol 2 and immediately centrifuge 8 min at 500 × *g*, ??°C.
2. Aspirate supernatant (do not decant, in order to prevent excessive cell loss), and resuspend the cell pellet in 300 µl PBS.
3. Transfer 100 µl of the cell suspension to a 12 × 75–mm polystyrene tube.

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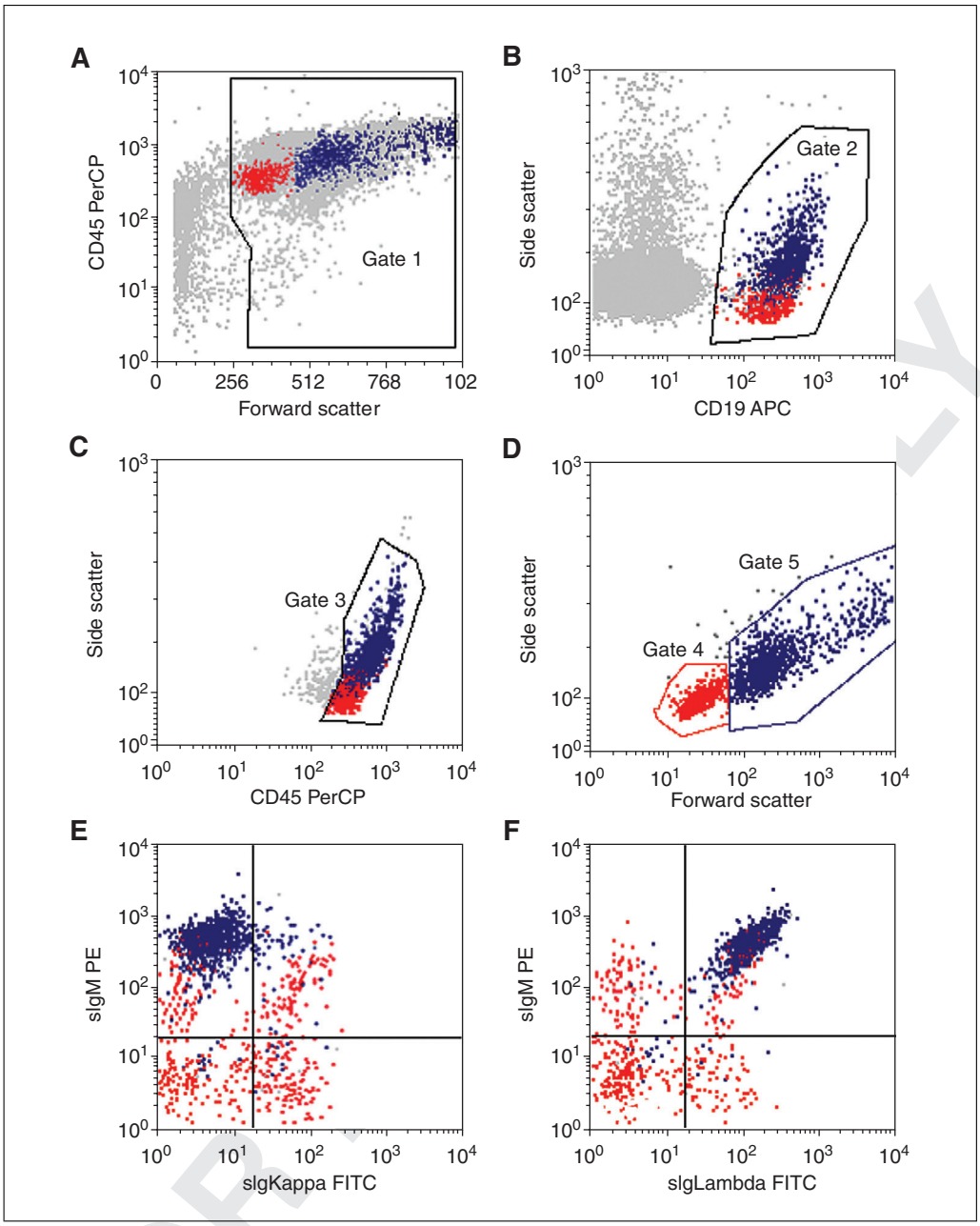

Figure 6.25.1 Example of a 4-color flow cytometric analysis for B lymphocyte clonality in CSF. Each dot represents a single cell. The units on the *x*- and *y*-axes are relative fluorescence intensity. For analysis, debris and non-leukocyte events were excluded by gating on forward scatter (FSC) and CD45 [gate 1, panel (**A**)]. B cells were gated using the lineage-specific marker CD19 and side scatter (SSC) [gate 2, panel (**B**)]. Next, mature B lymphocytes were defined by plotting events that meet criteria for both gates 1 and 2 in a CD45 versus SSC dot plot and selecting events with a low SSC and high CD45 expression [gate 3, panel (**C**)]. Based on difference in their physical parameters, two mature B lymphocyte populations could be detected, colored red [gate 4, panel D] and blue [gate 5, panel D]. Analysis of clonality revealed that the green dots are a population of normal B cells with polyclonal expression of IgM heavy and Kappa/Lambda light chains [red dots, panels (**E**) and (**F**)], while the blue dots represent a large population of monoclonal B cells expressing IgM heavy and Lambda light chains, compatible with large B cell lymphoma. [blue dots, panels (**E**) and (**F**)]. For the color version of this figure go to *http://www.currentprotocols.com*.

4. Add the exact amount of each properly titrated monoclonal antibody (MAb) to the tube according to the appropriate staining panel (see MAb panels; Tables 6.25.1-6.25.5), and mix gently.

   Optionally, 5 μl of the cell suspension may be taken prior to staining to perform a cell count using a hemacytometer (APPENDIX 3A) or a Fuchs-Rosenthal chamber to adjust cell concentration.

5. Incubate 15 min at room temperature in the dark.

6. Add 2 ml PBS and mix gently.

   If the sample is contaminated with peripheral blood and erythrocytes are a major population, lysis will be necessary. Samples can be lysed by adding 2 ml of ammonium chloride lysing solution (APPENDIX 2A) instead of PBS, then incubating 10 min at room temperature.

7. Centrifuge 5 min at 500 × *g*, ??°C, and aspirate the supernatant.

8. Add 250 μl of PBS containing 1% PFA. Mix gently.

Perform data acquisition and analysis

Acquire and analyze data immediately after sample preparation is completed. If the CSF sample does not show infiltration by neoplastic cells, the process should be repeated with the remaining volume of CSF sample and an identical MAb reagent combination to increase the sensitivity of the assay. In contrast, if a suspected population is identified in the first staining, a more extensive phenotypic characterization will result from staining with subsequent reagent panels and previous histological and/or immunophenotypic investigations.

9. Clean flow cytometer prior to acquisition with rinse solution for 5 min. Wash the flow cytometer with flow buffer for 5 min.

10. Acquire all cells or a minimum of 100.000 leukocytes/nucleated cells. Include the time parameter, which should be used to gate out events generated by air bubbles when tube runs empty.

11. Exclude debris and non-leukocyte events from analysis by gating on forward light scatter (FSC) and preferably CD45.

    Blast cells and myelomatous plasma cells may have CD45 negative/dim expression, particularly in B cell precursor acute lymphoblastic leukemia.

12. Select cells of interest using a lineage-specific marker and side scatter (SSC) and preferably a dual-anchor gating strategy (Nicholson et al., 1996) using CD45 versus SSC. Analyze un-gated data as well.

13. Use normal lymphocytes, monocytes, and neutrophils within the CSF sample as an internal control for negative or positive antigen expression.

    a. Analyze B cell data for abnormal patterns of antigen expression and/or light scatter characteristics (large-cell lymphoma) within a pan-B antigen (preferably CD19; in some occasions CD19 is weak or negative and may be replaced by CD20 and/or CD22). Analyze light-chain expressions within the abnormal population in combination with CD19 (CD20 and/or CD22) and patient-specific antigen or heavy-chain expression as well as light-scatter characteristics (Fig. 6.25.1: example of B-NHL gating strategy). Analyze clusters of cells thus identified for light-chain expression.

       Populations are classified as monoclonal (or showing clonal excess) when a surface immunoglobulin (sIg) Kappa/Lambda ratio below 0.25 or above 4 is observed, provided that enough (n > 100) sIg+ B cells have been measured.

       Some cases, i.e., diffuse large B cell lymphoma, may be light-chain negative.

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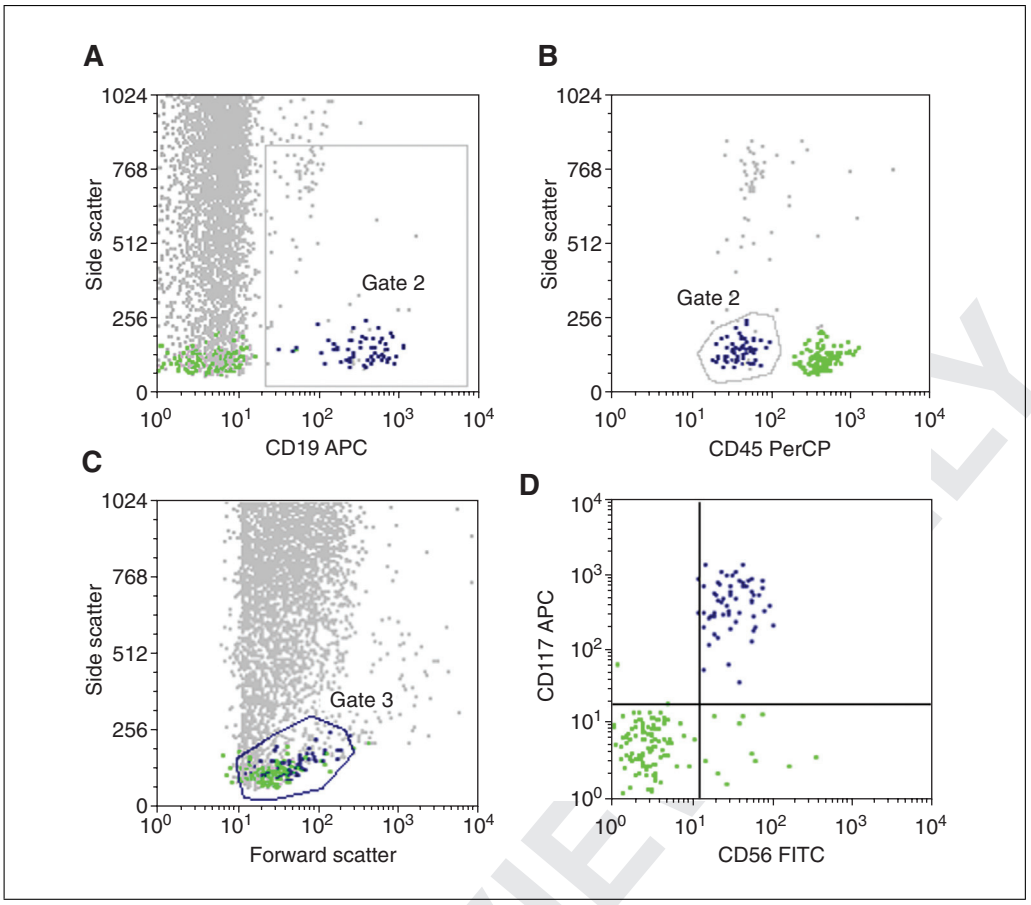

Figure 6.25.2 Example of a 4-color flow cytometric analysis of AML localization in CSF. Each dot represents a single cell. The units on the *x*- and *y*-axes are relative fluorescence intensity. A small population of myeloid blasts (0.3% of nucleated cells) could be detected by their expression of CD117 [gate 1, blue, panel (**A**)] and by sequential gating for their expression of $CD45^{dim}$, side $scatter^{low}$ [panel (**B**), gated on gate 2] and $FSC^{intermediate}$ [panel (**C**), gated on gates 1 through 3]. The LAP of the myeloid blasts was confirmed by coexpression of CD56 using lymphocyte events (green) as an internal negative control [panel (**D**)]. For the color version of this figure go to *http://www.currentprotocols.com*.

b. Analyze T cell data for abnormal patterns of antigen expression as well as light-scatter characteristics based on the known leukemia associated immunophenotype (LAIP; San Miguel et al., 1997).

c. Analyze leukemia data for clusters with an immunophenotype of blasts using CD45 negative/dim expression, SSC, and a lineage-specific and/or immature antigen expression marker (e.g., CD34) according to their LAIP, using a sequential gating strategy (*UNIT 6.4*)

Figures 6.25.2 and 6.25.3 illustrate examples of AML and B-ALL gating strategies.

14. Classify clusters of more than 25 events fulfilling the above criteria as positive, clusters of 10 to 25 events as suspicious, and clusters of less than 10 events as negative.

Note that the sensitivity and minimum number of events to define CNS involvement may vary depending on the number of parameters simultaneously assessed. The numbers given are typically used for 4-color assays.

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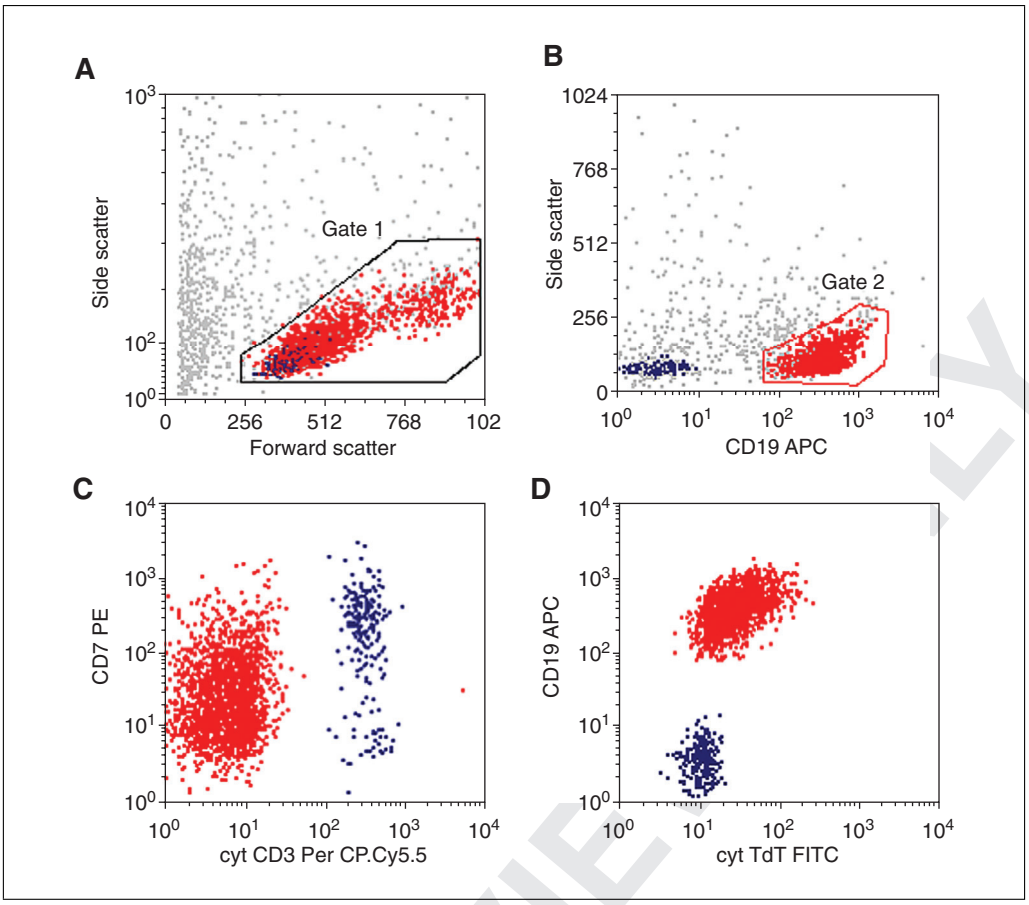

Figure 6.25.3 Example of a 4-color flow cytometric analysis of CNS involvement of B cell precursor ALL in CSF. Each dot represents a single cell. The units on the *x*- and *y*-axes are relative fluorescence intensity. For FCM immunophenotyping analysis, debris and non-leukocyte events (gray) were excluded by gating (Gate 1) in a forward scatter versus side scatter bivariate dot plot [panel (**A**)]. A population of B cell lymphoblasts could be detected by their expression of CD19 [gate 2, red, panel (**B**)], CD45^{dim} (data not shown), and their coexpression of cytoplasmic TdT [panel (**D**)] using CD3-positive lymphocyte events (blue) as an internal negative control [panels (**C**) and (**D**)]. For the color version of this figure go to *http://www.currentprotocols.com*.

COMBINED STAINING FOR SURFACE AND INTRACELLULAR ANTIGENS

SUPPORT PROTOCOL 1

The following method was developed to allow the simultaneous detection of surface and cytoplasmic and/or nuclear antigens. The procedure utilizes the Fix & Perm kit from Invitrogen, but other manufacturer's products may give equivalent results.

Additional Materials *(also see Basic Protocol)*

Surface antibody cocktail, titrated (see Strategic Planning and Tables 6.25.1-6.25.5)
Cytoplasmic antibody (cocktail), titrated (see Strategic Planning and Tables 6.25.1-6.25.5)
Fix & Perm kit from Invitrogen, Intrastain kit from Dako, or Intraprep from Beckman Coulter

Label cell surface antigens

1. Take 100 µl of CSF sample (as prepared in Support Protocol 2) and place in a 12 × 75–mm polystyrene tube.
2. Add the exact amount of each properly titrated surface MAb (see MAb panels; Tables 6.25.1-6.25.5) to the tube and mix gently.

   For this step, use an antibody cocktail lacking reagents directed against cytoplasmic or nuclear antigens.

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3. Incubate for 15 min at room temperature in the dark.

Fix cells

4. Add 2 ml PBS to the tube and mix gently.
5. Centrifuge 5 min at 500 × *g*, ??°C, and aspirate the supernatant.
6. Add 100 µl fixation solution (from kit) and mix well.

   CAUTION: *The fixative reagent contains formaldehyde and should be handled with appropriate precautions.*

7. Incubate for 15 min at room temperature in the dark.

Permeabilize cells

8. Add 2 ml PBS to the tube and mix gently.
9. Centrifuge 5 min at 500 × *g*, ??°C, and aspirate the supernatant, leaving no more than 100 µl in the tube.
10. Add 100 µl of permeabilization solution (from kit).

Label cytoplasmic or nuclear antigens

11. Add the exact amount of each properly titrated cytoplasmic or nuclear MAb to the tube and mix well.

    For this step use an antibody cocktail containing only reagents directed against cytoplasmic or nuclear antigens.

12. Incubate for 15 min at room temperature in the dark.
13. Add 2 ml PBS to the tube and mix gently.
14. Centrifuge 5 min at 500 × *g*, ??°C, and carefully aspirate the supernatant without disturbing the pellet, to minimize cell loss.
15. Add 250 µl of PBS containing 1% PFA. Mix gently.

Perform data acquistion and analysis

16. Acquire and analyze immediately after sample preparation is completed. For data acquisition and analysis, see Basic Protocol, steps 9 to 14.

ALTERNATE PROTOCOL

ENUMERATION OF ABSOLUTE LEUKOCYTE SUBSET COUNTS

The following method was developed to obtain absolute numbers of both normal and neoplastic cells in cerebrospinal fluid by adding counting beads to the sample before acquisition. In spite of the potential loss during concentration/centrifugation steps, the knowledge of frequency and distribution of the major cell populations can be of great help in evaluating the sample.

Additional Materials *(also see Basic Protocol)*

Aqueous suspension of microspheres (Perfect Count form Invitrogen; alternatives include FlowCount from Beckman Coulter, CytoCount from Dako, and Cyto-Cal Count Control from Duke Scientific)

Label cell surface antigens

1. Add 3 to 5 ml PBS to the CSF sample obtained in Support Protocol 2 and immediately centrifuge for 8 min at 500 × *g*, ??°C.
2. Aspirate supernatant (do not decant, in order to prevent excessive cell loss) and resuspend cell pellet in 300 µl PBS.

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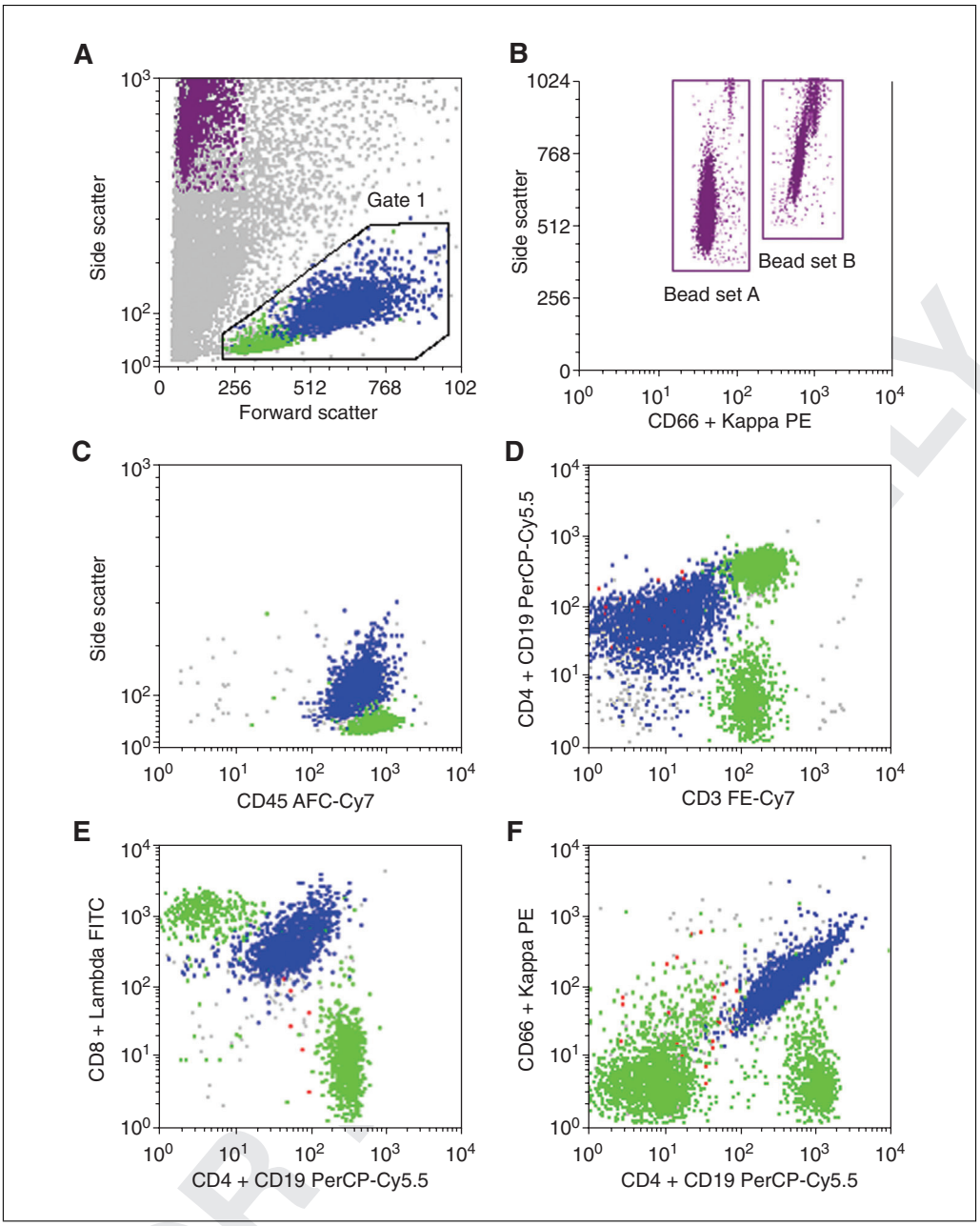

Figure 6.25.4 Example of a 6-color flow cytometric analysis of a normal/reactive CSF sample. For absolute count evaluation, microspheres are used [purple dots in panel (**A**) and panel (**B**)]. Each dot represents a single cell. The units on the *x*- and *y*-axes are relative fluorescent intensity. For FCM immunophenotyping analysis, debris and non-leukocyte events (gray) were excluded by gating (Gate 1) in a forward scatter versus side scatter (panel A) and a side scatter versus CD45 antigen expression bivariate dot plot [panel (**C**)]. Panels [(**D**) to (**F**)] show the identification of the two major cell subsets present in the sample: total T lymphocytes ($CD45^+$, $CD3^+$, $CD4^+$, and/or $CD8^+$ events; blue dots) and monocytes ($CD45^+$, $CD3^{neg}$, $CD4^{dim}$, $CD14^+$, autofluorescent events; green dots). B lymphocytes ($CD45^+$, $CD3^{neg}$, $CD14^{neg}$, $CD19^+$; red dots) are sporadically detectable and polyclonal. For the color version of this figure go to *http://www.currentprotocols.com*.

3. Transfer one-third (100 µl) of the cell suspension to a 12 × 75–mm polystyrene tube.
4. Add the exact amount of each properly titrated MAb to the tube according to the appropriate staining panel (see MAb panels, Tables 6.25.1-6.25.5), and mix gently.
5. Incubate 15 min at room temperature in the dark.

6. Add 2 ml PBS and mix gently.

   If the sample is contaminated with peripheral blood and erythrocytes are a major population, lysis will be necessary. Samples can be lysed by adding 2 ml of ammonium chloride lysing solution instead of PBS, then incubating 10 min at room temperature.

7. Centrifuge 5 min at 500 × *g*, ??°C, and aspirate the supernatant.

8. Add 100 µl of PBS and 100 µl of microsphere suspension. Mix gently.

Perform data acquisition and analysis (see also Fig. 6.25.4)

Acquire and analyze data immediately after sample preparation is completed. If the CSF sample does not show infiltration by neoplastic cells, the process should be repeated with the remaining volume of CSF sample and an identical MAb reagent combination to increase the sensitivity of the assay. In contrast, if a suspected population is identified in the first staining, a more extensive phenotypic characterization will result from staining with subsequent reagent panels and previous histological and/or immunophenotypic investigations.

9. Acquire data using the same acquisition and gating strategy described in the Basic Protocol, steps 9 to 14.

10. Analyze the number of events for each of the normal and neoplastic cell subset to be reported in absolute counts.

11. Define the number of acquired microsphere events for the tube with a gate selection as described in the manufacturer's instructions.

12. Calculate the absolute number of each cell subset in a tube using the following formula:

$$\frac{\text{events in cell subset gate}}{\text{events in microsphere gate}} \times \frac{\text{microspheres added per tube}}{\text{volume of CSF sample (µl)}} \times \text{correction factor} = \text{cells/µl}$$

The total number of microspheres added per tube can be calculated as follows: volume of microspheres added to the tube (i.e., 100 µl) × microsphere concentration (microspheres/µl) provided by the manufacturer.

Volume of CSF sample refers to the collected volume of CSF sample without stabilization buffer.

Correction factor will depend on the portion of the cell suspension used for a tube in step 9 (e.g., 100 µl of a 300 µl cell suspension will result in a correction factor of 3).

SUPPORT PROTOCOL 2

SAMPLE COLLECTION

[? introduction?]

Materials

Patient/CSF donor
Stabilization medium 1—complete RPMI (*APPENDIX 3B*) containing 5% FBS—*or* stabilization medium 2—Transfix (Cytomark, *http://www.cytomark.co.uk*)

Equipment for lumbar puncture
15-ml tubes

1. Collect ≥2 ml of CSF fluid by lumbar puncture. Place sample at 4°C.

   Sample should be processed within 1 hr or stabilized in stabilization medium 1 or 2.

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2a. *Using stabilization medium 1:* Collect the abovementioned 2-ml CSF sample directly into a sterile 15 ml tube containing 2 ml stabilization medium 1. Store up to 18 hr at 4°C.

2b. *Using stabilization medium 2:* Collect the 2-ml CSF sample directly into a tube containing 0.4 ml stabilization medium 2 (for 1/5 ratio of stabilization medium to CSF). Store up to 48 to 72 hr at 4°C.

COMMENTARY

Background information

Diagnosis of leptomeningeal involvement by a hematological malignancy has great prognostic and therapeutic implications both in symptomatic and asymptomatic patients at high risk for central nervous system (CNS) disease. Among others, myeloid and B lymphoid neoplasias are the most common hematological malignancies showing leptomeningeal infiltration. The reported frequency of leptomeningeal disease in non-Hodgkin's lymphoma (NHL) ranges from 2% to 27% in several patient cohorts (Montoto and Lister, 2005). Such variability may be explained by the different histological subtypes, the criteria used to define CNS infiltration, the inclusion of newly diagnosed versus recurrent patients, and/or the use of CNS prophylaxis (Montoto and Lister, 2005). Most frequently, B-NHLs that involve the CNS tend to be high-grade, aggressive B cell neoplasias (e.g., Burkitt lymphoma and diffuse large B cell lymphoma with extranodal involvement), with a CNS infiltration rate of between 5% and 8% to 17% of the cases (Feugier et al., 2004, Montoto and Lister, 2005). In contrast, CNS infiltration in patients with low-grade lymphomas is considerable lower (<5% of the cases; Montoto and Lister, 2005). Although on most occasions CNS involvement is a secondary tissue-involvement site of aggressive lymphomas, it can also be their primary localization. In these latter cases, diagnosis is suspected on the basis of neurological symptoms and/or suggestive neuroimaging characteristics. Previous reports have shown that, in addition to the histological subtype, there are several clinical characteristics which show a clear association with CSF involvement (e.g., presence of two or more extranodal sites, increased lactate dehydrogenase serum levels, and a high International Prognostic Index). These criteria are currently being used to select patients for CNS prophylaxis; however, the predictive value of these parameters for developing CNS disease still remains limited due to both a relatively low sensitivity and specificity (Montserrat et al., 1996; Van Besien et al., 1998; Hollender et al., 2002).

Diagnosis of CNS disease is frequently suspected based on the presence of clinical manifestations of CNS involvement and the results of investigations with neuroimaging techniques. Morphological identification of the presence of malignant hematopoietic cells in the CSF currently represents the method of choice for the definitive diagnosis of CNS involvement both at diagnosis and recurrence of the disease, with direct therapeutic and prognostic implications (Van Besien et al., 1998; Schinstine et al., 2006). Despite the high specificity of conventional cytology (CC), this technique is associated with a relatively limited sensitivity, with up to 20% to 60% false-negative results (Glass et al., 1979; Wasserstrom et al., 1982; Kaplan et al., 1990; Freilich et al., 1995; Roma et al., 2002); interpretation of the cytological findings, may be difficult because of paucity of cells in CSF and the morphological similarities between benign and malignant cells (Windhagen et al., 1999; DeAngelis and Cairncross, 2002; Hegde et al., 2005).

In recent years, several attempts have been made to increase the diagnostic accuracy of conventional cytology through the evaluation of diagnostic techniques that are complementary to the study of CSF samples, including the combined use of morphological, immunophenotypic, molecular, and cytogenetic techniques. Interestingly, flow cytometry (FCM) has long been considered to have serious limitations for the study of CSF samples, mainly related to the low cell counts observed (Subira et al., 2002). However, recent studies clearly show that flow cytometry could be more sensitive than conventional cytology for the detection of CSF involvement by neoplastic cells (French et al., 2000; Subira et al., 2002; Hegde et al., 2005; Bromberg et al., 2007), with the combined use of CC and FCM leading to a significant increase in the rate of CSF involvement in both newly diagnosed and relapsed/treated hematological malignancies (Finn et al., 1998; Hegde et al., 2005; Nückel et al., 2006; Bromberg et al., 2007). Of note, under these conditions, flow

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cytometric analysis of CSF samples has to be applied to paucicellular samples which contain a limited number of cells with a rapidly decreasing viability (Subira et al., 2002) and specific sample preparation protocols and analytical approaches should be used, such as those described in this unit.

Critical Parameters

Sample quality

Cellular degeneration of CSF will significantly become evident within 1 hr of collection. Therefore, samples should be transported at ambient temperature to the testing site as soon as possible and processed with minimal sample handling, or they may be immediately stabilized as described in Support Protocol 2.

Blood contamination

Red cells reflect either central nervous system bleeding or a traumatic puncture. If the puncture is traumatic, the number of peripheral blood leukocytes (white blood cells, WBC) added to the sample can be calculated from the CSF red cell count (red blood cells, RBC) and the relative number of leukocytes and erythrocytes in blood:

$$WBC_{added} = WBC_{blood}/RBC_{blood} \times RBC_{csf}$$

An easier way to identify blood contamination can be done empirically by examining the number of erythrocytes per leukocyte: more than 1000 erythrocytes per one leukocyte means blood contamination.

In case of secondary leptomeningeal infiltration, detection of a small population of neoplastic cells in a blood-contaminated CSF sample could only be interpreted as CNS involvement when these cells are not detected in a blood sample of the same drawing date.

Removal of free immunoglobulin

CSF samples can contain significant amounts of free immunoglobulins (Igs). Failure to adequately remove free immunoglobulins results in binding of labeled anti-human immunoglobulin antibodies (e.g., anti-Kappa) to free antigen in the sample rather than to the cell-associated antigens of interest, and a reduced or absent reactivity with anti-Hu-Ig reagents is observed. Diluting the sample with an equal amount of washing buffer during the first centrifugation/concentration step as described in this protocol will be sufficient to prevent binding of free Igs by CSF samples.

Enumeration of immunologically defined rare cell populations

CSF samples normally contain a limited number of cells, and frequently the neoplastic cells constitute a small fraction of the total number of leukocytes. The smaller this fraction, the greater need for discrimination of bona fide events from background signals (noise). For accurate detection of rare events, background signals must be substantially less than the frequency of the events sought. Thus, for rare cells at a frequency of 0.01% of total cells, the cytometric assay would need to be greater than 99.99% free of noise. Background noise is generally due to a variety of factors that yield fluorescent signals, or at least signals perceived by the cytometer to be fluorescent, in the absence of antibodies or fluorescent probes specific to the cells of interest. Multicolor staining in combination with sequential gating strategies has been proven useful for the identification of rare cells. In rare-event analysis, large numbers of cells must be counted to obtain statistically meaningful numbers of rare cells. Rare events typically follow a Poisson distribution, according to which their CV (in %) is $100 \times n^{1/2}/n$, in which n = number of events fulfilling the flow cytometric criteria of the rare events under study. Hence, their CV will vary proportionally to the square root of the number of rare events acquired. Another source of background in rare-event analysis is carryover of cells in the cytometer from previous experiments. Before rare-event analysis on any cytometer, extensive cleaning and washing is recommended to remove residual cells and particles. The extent and nature of this cleaning will differ with the make and model of cytometer.

Anticipated Results

The results that immunophenotypic analysis of CSF will provide depend on the combination of reagents used and the populations being identified. If the principles outlined above are followed, discrete populations of neoplastic cells can be separated from normal leukocytes in CSF samples. Sensitivity of the assay will be increased by using a higher number of measurements per cell and by optimal use of all available parameters of the flow cytometer, as well as the total volume of CSF analyzed. Evaluated leukocyte counts can be present in numerous conditions (infection, intracranial hemorrhage, or metabolic disorders). Only in the case of CSF involvement in leukemia will blasts be detectable in spinal fluid. In normal

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Table 6.25.6 Normal Values of Lymphocyte Subsets in Adults $n = 27$ (de Graaf et al., submitted)

Lymphocyte subset in CSF	Median (cells/μl)	5th-95th percentile (cells/μl)
T cells	0.46	0.2-2.02
CD4+ T cells	0.34	0.12-1.36
CD8+ T cells	0.13	0.06-1
B cells	0.005	0-0.034
NK cells	0.011	0.002-0.058
NKT cells	0.005	0-0.037

CSF samples B lymphocyte levels are close to detection level (see normal values of lymphocyte subsets in Table 6.25.6), but will be increased in the case of leptomeningeal disease resulting from B cell lymphoma.

Sensitivity of detection of T cell lymphomas will strongly depend on the LAIP, which discriminates between normal and neoplastic T cells.

Time Considerations

The Basic as well as the Alternate Protocol take roughly 45 min to obtain the results of the first step, and the total analysis will take up to 2 hr. The use of Support Protocol **1** increases the processing time by 45 min to up to 3 hr.

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